STUDIES OF FERTILIZATION, X.

The Effects of Copper Salts on the Fertilization Reaction in Arbacia and a Comparison of Mercury Effects.

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1. Introduction.

Copper salts are known to have a profoundly injurious effect on many organisms even in high dilutions. They are also known to form compounds with ferments and proteins (on one side of the isoelectric point). It might therefore be expected in advance that they would have very definite effects in the delicate reactions of fertilization, which might be used advantageously in the analysis of these reactions, the precise character of which is still a matter of dispute.

The results reported were obtained in the summers of 1920 and 1921 at the Marine Biological Laboratory at Woods Hole. They have led me to the conclusion that the fertilization reactions in the sea-urchin are due primarily to activation of a ferment-like substance contained in the cortex of the egg which is presumably identical with the fertilizin of my earlier studies. The method of approach is quite new in fertilization studies, but has been previously used in ferment studies, as I discovered after the conclusion of my experiments. Certain points of comparison between inactivation of ferments by salts of heavy metals and inactivation of the fertilization reaction were found.

As soon as we adopt the conclusion that fertilization is essentially activation of a substance, contained in the cortex of the egg, by the spermatozoön (Lillie, 1914, 1919), the way is open for study of the properties of this substance by means of inhibitors of fertilization in general. If we were to determine the qualitative and quantitative range of such inhibitors we would be in possession of a list of properties of the postulated substance that

should enable us to classify it very closely. The present paper is a contribution to this program.

II. Methods.

The eggs of the sea-urchin, used in these experiments, should be washed in two or three changes of sea-water to rid them of blood or tissue secretions, and they should not be used for more than about three hours after washing. The quantities used should be small and constant for all experiments of a set; as a practical matter not more than two drops of eggs from the layer on the bottom of the container to 10 c.c. of sea-water. In more delicate experiments egg concentration should be expressed as number of eggs per c.c. of sea-water.

Sperm concentration may be expressed in terms of dilution of the dry sperm as I have done previously. For instance, one drop (0.1 c.c.) of dry sperm may be added to 25 c.c. of sea-water, and one drop (0.04 c.c.) of this suspension used to fertilize one drop of eggs in 7.5 c.c. sea-water. This may be expressed as one drop of 1:25:7.5 c.c. Such an insemination is abundant but not "heavy." It is equal to about 1.5 "units sperm concentration" of study VIII. Sperm concentration is given for all the experiments. A 1:25 sperm suspension should be prepared immediately before using and should not be used for more than about five minutes, as its fertilizing power diminishes rapidly. The main point is to make sure that the inseminations of a series are identical or comparable.

A solution of 1 per cent. by weight of c.p. copper chloride in distilled water was made; 0.1 per cent. in distilled water was made from this, and the various concentrations actually employed in the experiments were made immediately before each experiment by adding a definite number of drops of the 0.1 per cent. solution to 100 c.c. of sea-water, using a pipette graduated in hundredths of a c.c. on which the actual amount added was read off. The strength of copper chloride most commonly employed was 5 drops of 0.1 per cent. in 100 c.c. of sea-water. The readings of the 5 drops ranged around 0.19 c.c.; in round numbers this was calculated in as 0.2 c.c. and the amount of $CuCl_2$ in such a

solution is accordingly given as one part in 500,000. This is approximately 1/67,000 N, but on account of the alkalinity of the sea-water the effective concentration must be somewhat less.

In some early experiments a stock solution of copper sulphate in sea-water was used. The strength of this declined rapidly and it was used only in experiments 1 to 9. Its physiological effects are the same as copper chloride.

For each experiment there are two standard controls, viz.: (1) the *copper control*, *i.e.*, an identical insemination in copper chloride of the same concentration, and (2) *gamete control*, *i.e.*, an identical insemination in sea-water. Special experiments had in addition their special controls.

III. Experiments.

1. *The Phenomenon of Copper Inhibition. Relations between copper concentration and sperm concentration.*

The presence of one part of copper chloride in 500,000 parts of sea-water will completely inhibit fertilization of the eggs of *Arbacia*. Previous exposure of the gametes to the action of the copper chloride is not necessary for this result; if eggs and sperm are dropped simultaneously into the copper-containing sea-water, and mixed at once, no reaction occurs, though the sperm are as active as in normal sea-water. With most batches of eggs the inhibition is complete even when the amount of sperm used is several times what is necessary to fertilize 100 per cent. of the eggs in normal sea-water.

Though the eggs may be bombarded by hundreds of spermatozoa each, they do not usually give the first beginnings of the fertilization reaction; but, if any egg does so, fertilization is carried through to completion in the copper solution, and the egg segments. The inhibition is thus an "all or none" effect, as is shown more fully beyond.

The relations between copper concentration and sperm concentration are given in Table I. If the concentration of the copper is much greater than one part of copper chloride in 500,000 parts of sea-water, the inhibition may be regarded as complete for all sperm concentrations.

TABLE I.

RELATIONS BETWEEN COPPER CONCENTRATION AND SPERM CONCENTRATION. Time of exposure before fertilization = 0. Percentages are of eggs that segmented.

In these experiments the eggs were fertilized with definite concentrations of sperm (horizontal lines) in sea-water containing copper chloride to the amount of 1 part in 2,500,000, one part in 1,250,000, etc., to one part in 416,666 (vertical columns).

Sperm Concentration.	Sperm Concentration Units. See Study VIII.	1 / 2,500,000 $CuCl_2$	1 / 1,250,000 $CuCl_2$	1 / 833,333 $CuCl_2$	1 / 625,000 $CuCl_2$	1 / 500,000 $CuCl_2$	1 / 416,666 $CuCl_2$	Control No $CuCl_2$	Number of Experiment.
One drop 1 : 25 : 7.5 c.c.	1.5	50%	7%	0	0	0		100%	Exp. 35
Five drops 1 : 25 : 7.5 c.c.	7.5	100%	7%	50%	0	0		100%	Exp. 35
One drop 1 : 25 : 7.5 c.c.	1.5			5%	0	0	0	100%	Exp. 36
Five drops 1 : 25 : 7.5 c.c.	7.5			2%	1%	0	0	100%	Exp. 36
Two drops 1 : 5 : 7.5 c.c.	15			5%	1%	0	0	100%	Exp. 36
Five drops 1 : 5 : 7.5 c.c.	37.5			12%	2%	0	0	100%	Exp. 36
One drop 1 : 32 : 7.5 c.c.	1					0		85–90%	Exp. 14
Two drops 1 : 32 : 7.5 c.c.	2					0			Exp. 14
Four drops 1 : 32 : 7.5 c.c.	4					0			Exp. 14
Eight drops 1 : 32 : 7.5 c.c.	8					0			Exp. 14
Two drops 1 : 4 : 7.5 c.c.	16					0.2%		100%	Exp. 14
Four drops 1 : 4 : 7.5 c.c.	32					0.2%			Exp. 14
Eight drops 1 : 4 : 7.5 c.c.	64					0.5%			Exp. 14
Sixteen drops 1 : 4 : 7.5 c.c.	128					3%			Exp. 14

It will be noticed that inhibition is marked even at as low a concentration as one part of copper chloride in 2,500,000 parts of sea-water at a normal sperm concentration. Most of the experiments to be described were done with 1/500,000 copper chloride, at which concentration no eggs fertilize at normal sperm concentrations.

If much higher concentrations of sperm are used small percentages of eggs, varying somewhat in different experiments, may fertilize. (See last items of Table I.) There is thus a certain virtue in mass action of the sperm in the presence of this inhibitor of fertilization; this is somewhat difficult to understand, because only one spermatozoön penetrates usually. If we regard the spermatozoa, or some substance borne by them, and the copper as reacting with the same substance of the egg, it can be understood how the sperm substance could replace the copper in some cases when present in excess; or it may be possible that excess of sperm protects the eggs to a certain extent by combining with the copper and thus reducing the amount acting directly on the eggs.

2. *Reversibility of Copper Inhibition.*

Eggs that have been exposed to copper, whether in the presence of sperm or not, may be fertilized after return to sea-water provided that the exposure has not been long enough to injure their vitality too much. In other words, the inhibition by copper is reversible, and the phenomenon is to be regarded as one of inactivation of a substance, not of its destruction. In this respect, the phenomenon is precisely like the inactivation of an enzyme by mercury or copper salts, which is similarly reversible, and which has been shown to be due to a combining of the ions in question with constituents of the enzyme solution (v. Euler and Svanberg, 1920).

3. *Effect of Copper Chloride on Spermatozoa and Eggs Separately.*

A suspension of spermatozoa in 1/500,000 copper chloride has been tested up to 8 minutes exposure of the spermatozoa by fertilizing eggs in sea-water without any noticeable diminution in the fertilizing power of the sperm. The fertilizing power of a sperm suspension made in 1/25,000 copper chloride in sea-

water began to fall off rapidly after two minutes; after four minutes only 5 per cent. of the eggs fertilized.

Eggs, on the other hand, show an effect of exposure to 1/500,000 copper chloride from 10 seconds exposure on, if transferred and fertilized in sea-water. The effect is seen first in poor viability, then in increase of polyspermy (after 1 minute exposure), but they may form membranes even after 8 minutes exposure. If exposed to 1/25,000 copper, 50 per cent. will not fertilize at all after 1 minute. The eggs in short are much more sensitive to the copper than the sperm.

Unfertilized eggs left in 1/500,000 copper chloride in sea-water begin to show visible signs of injury after about two hours at (approximately) 20° C. The surface of the egg beneath the membrane first appears roughened, then by degrees a perivitelline space appears containing a fluid stained red by escaping pigment; a true cytolysis involving a "laking" effect due to destruction of the plasma membrane has occurred. At this time, except for the presence of pigment in the perivitelline fluid, the eggs look as though they were provided with fertilization membranes. Following this, cytoplasmic buds appear, the nucleus swells and the egg disintegrates very gradually.

4. *Effect of Copper Chloride on Fertilized Eggs.*

(*a*) If eggs are fertilized in normal sea-water and transferred to 1/500,000 copper chloride in sea-water two or more minutes after insemination, they continue their development for several hours, up to a late cleavage stage at least; but the copper acts as a slow poison, so that the eggs rarely reach a swimming stage. Eggs may segment in 1/250,000 and 1/125,000 copper chloride if transferred 5 or more minutes after insemination in sea-water; but their rate of death is naturally increasingly rapid. In general the length of life of fertilized eggs is about the same in any concentration used, whatever be the time of transfer before cleavage. Successive stages of fertilization after the first five minutes do not appear to vary notably in their sensitiveness to $CuCl_2$ within the range explored. The effect of $CuCl_2$ on the stages of fertilization after the first few minutes is thus a progressive poisoning varying in rate with concentration.

There is thus an extraordinary contrast between fertilized and unfertilized eggs in their sensitiveness to copper; it is obvious that copper has an effect that belongs to a separate order of sensitiveness on the early stages of fertilization as compared with later stages.

(*b*) The question thus arises as to the time limits of the early inhibiting copper effect. A number of experiments were performed to test this point, two of which are presented in Tables II. and III. In these experiments the eggs were inseminated in sea-water and then transferred at intervals to the copper solution. Samples of the eggs shown in Table III. were transferred back to sea-water after fourteen minutes in the copper solution to test their viability.

TABLE II.

(EXP. 12.) EFFECTS OF COPPER CHLORIDE ON EGGS INSEMINATED IN SEA-WATER. A very heavy insemination was used in this experiment: two drops of 2 : 5 : 7.5 c.c.

Transfers to 1/500,000 $CuCl_2$.	Per Cent. Segmented.	Remarks.
(*a*) 4 seconds after insemination.....	10% +	Irregular cleavage.
(*b*) 6 seconds after insemination.....	10% +	Irregular cleavage.
(*c*) 10 seconds after insemination.....	80–90%	Irregular cleavage.
(*d*) 20 seconds after insemination.....	80–90%	Irregular cleavage.
(*e*) 30 seconds after insemination.....	100%	Irregular cleavage.
(*f*) 60 seconds after insemination.....	100%	Irregular cleavage.
(*g*) Control in sea-water.....	100%	Normal.
(*h*) Control inseminated in the Cu solution.....	1%	Irregular cleavage.

Here we must note two results particularly:

(1) Eggs that have begun fertilization reaction before transfer to the copper solution go through to cleavage, while those that have not begun it are instantaneously inhibited. Transfers made even four seconds after insemination (Table II.) include a considerable proportion of eggs (varying with sperm concentration in various experiments from 5 to 25 per cent.) which complete the fertilization and segment. The percentage rises to normal with increase of time before transfer.

(2) Eggs transferred from normal insemination to the copper chloride within the first two minutes do not give a complete

membrane reaction. The membranes of such eggs are "narrow," or better, the perivitelline space separating egg and membrane is narrow, often extremely so, down to the point of invisibility. The viability of such eggs is bad even if they are returned to sea-water again within a few minutes (Table III.). Indeed, the viability forms an ascending series to normal from 10 seconds up to 5 minutes. In other words, the fertilization reaction exhibits gradation in intensity according to the point of time at which the copper begins to operate, reaching normal at about two minutes with the best lots of eggs, and viability is proportional to fertilization intensity. There is no recovery from an initial subnormal reaction.

TABLE III.

(EXP. 37.) EFFECTS OF COPPER CHLORIDE ON EGGS INSEMINATED IN SEA-WATER. Insemination, one drop of 1 : 5 : 5. Eggs replaced in sea-water after 14 minutes' exposure to $CuCl_2$. This experiment came at the end of the season when the eggs were not in first class condition.

	Transfers to 1/500,000 $CuCl_2$.	Per Cent. Segmented.	Remarks.
(*a*)	10 seconds after insemination	15%	Membranes "narrow."
(*b*)	20 seconds after insemination	30%	Viability bad.
(*c*)	25 seconds after insemination	30%	
(*d*)	40 seconds after insemination	50%+	
(*e*)	60 seconds after insemination	60%	Membranes "narrow."
(*f*)	90 seconds after insemination	70%	Viability better.
(*g*)	2 minutes after insemination	80%	Viability still better. Not equal to control.
(*h*)	5 minutes after insemination	80%+	
(*i*)	10 minutes after insemination	90%	Equal to control.

The period of time involved in these subnormal effects is that of membrane formation or of cortical discharge.

We have, thus, three distinguishable effects of copper chloride at successive stages of fertilization:

1. To inhibit activation if present at insemination.

2. To reduce the intensity of the cortical discharge, if its action begins within the first two minutes, proportionally to the time of its operation.

3. A slow cumulative injurious effect thereafter which belongs to a different order of events from 1 and 2, and which does not become obvious until late cleavage.

The data then give rise to the hypothesis that there is an activable substance present in the cortex of the unfertilized egg for which copper possesses special affinity. Copper thus prevents the inception of activation when present before insemination; during membrane formation it causes a quantitative reduction of activation. Thereafter, this substance having been consumed, the specific copper effects are absent.

Copper affects primarily the *activation* of this substance, and presumably not its *operation*, or at least to a very much less extent. The quantitative effects of the first two minutes can be understood in terms of the respective amounts activated and unactivated at the time of exposure to copper.

Using our earlier term *fertilizin* for the activable substance of the egg we may now attempt to form a picture of what actually happens in the copper chloride sea-water.

When a given lot of eggs is inseminated normally there is an interval of time taken up by the meeting of the spermatozoa and the eggs. The length of this interval will naturally vary statistically for any given insemination, and will tend to be inversely proportional on the average to sperm and egg concentration. Fertilization proper begins after the actual agglutination of the spermatozoön to the surface of the egg, following a latent period of variable duration. The activation of the fertilizin is then begun and the egg becomes sterile to other spermatozoa. As Just ('19) expresses it, a "wave of negativity" sweeps over the surface of the egg from the point of attack of the successful spermatozoön. Now the rate of this wave is sufficiently rapid to prevent polyspermy even at high sperm concentration where the eggs are in their best condition; the wave must therefore be completed very rapidly. As a result of activation the egg engulfs the spermatozoön within one minute or less, and the fertilization membrane elevates beginning at the point of entrance of the spermatozoön (cf. Just). Thus the events are (1) agglutination of the spermatozoön to the egg, (2) latent period, (3) activation and sterilization, (4) penetration of the spermatozoön, (5) membrane formation.

It does not necessarily follow that all of the fertilizin is acti-

vated in the first wave of negativity; this wave may be entirely superficial and activation may thence extend into deeper layers of the cortex. When, therefore, eggs are inseminated in sea-water and then transferred within the first few seconds to the copper sea-water there will be a certain proportion in which the initial reaction has begun, and this proportion will be larger according as the sperm concentration is higher, as actual experiments show. The copper will, however, instantaneously check the activation of more fertilizin so that the eggs must operate in the copper sea-water with that portion already activated. Transfers at later stages will be successively less affected by the copper, as successively greater amounts of the fertilizin will have been activated before transfer, until all is in operation. When this point is reached, at membrane elevation, the other events of fertilization proceed as well in the copper sea-water as in normal sea-water.

If this form of interpretation is correct, we can perhaps see a little farther into the fertilization reaction. If activation of fertilizin follows immediately after agglutination of the spermatozoön to the egg, we would expect in the case of a heavy insemination that activation would have begun in all of the eggs within a few seconds, say 5 to 10, after insemination, because it is practically certain that in that space of time all eggs will have agglutinated spermatozoa. However, we notice in both of the tables and especially in Table III. that the proportion of eggs that segment does not suddenly rise to normal in a ten-second exposure. In some cases the rise is relatively sudden, as in Table II., in other cases relatively slow, as in Table III., and this corresponds to the physiological condition of the eggs as shown by the promptness of their behavior in normal fertilization. In other words, it is necessary to postulate a latent period after agglutination of the spermatozoön before activation begins, which varies with the physiological condition of the egg. This fact could be brought out only by use of a reagent like copper that instantaneously checks activation. When the wave of activation is begun it spreads flash-like over the surface, though this rate varies also with physiological condition as shown by the relation of the latter to

polyspermy. It is not necessarily the first spermatozoön that agglutinates that effects the initial discharge, but this will depend on physiological condition of the spermatozoön, and also perhaps on local differences of physiological condition on the surface of the egg.

5. *The Effect of Copper on Agglutination of Spermatozoa.*

The objection might be raised that copper inhibits by preventing agglutination of the spermatozoön to the egg, and not in the later activation of the fertilizin. The effect of copper on the agglutination reaction of the spermatozoa was therefore studied: Egg-water (of 1,600 agglutinating units) diluted 100 times or more with 1/500,000 copper chloride in sea-water has the same agglutinating effect on a sperm suspension in the same copper solution as when no copper is present either in the egg-water or sperm suspension. Agglutination will occur even in the presence of one part of copper chloride to 25,000 parts of sea-water, though more slowly. Copper chloride in the concentrations employed in the fertilization experiments has no noticeable effect on rate or duration of the agglutinating reaction.

The inhibiting action of copper must then occur after the agglutination of the spermatozoön to the egg, as was assumed in the preceding discussion.

6. *Protective Action of Egg-Water Against Copper.*

If copper chloride inhibits by combining with the fertilizin of the egg, then egg-water which contains fertilizin should protect against the inhibition of fertilization by deviating the copper from the fertilizin in the egg to that in the egg-water. Now it is known that egg-water contains a sperm-activating, a sperm-aggregating and a sperm-agglutinating substance (Lillie, '14, '19), and I have identified the sperm-agglutinating substance with fertilizin. These considerations led to experiments on the protective action of egg-water on the copper inhibition, in which definite positive results were obtained.

The egg-water is obtained by placing eggs in sea-water which receives their secretions. The strength of the egg-water depends on egg-concentration primarily, and also on time to a certain extent; it may be measured, as far as the sperm-agglutinating

constituent of the egg-water is concerned, by finding the greatest dilution at which it will cause visible agglutination in a sperm suspension. If the proportion of eggs to sea-water is about 1 to 4 in bulk, the egg-water from fresh eggs will usually stand dilution to about 1/1,600, and would therefore be accordingly rated as 1,600 units agglutinating strength. Other constituents of the egg-water are diluted at the same time naturally, but there is no present means of measuring these.

In a preliminary experiment (No. 21) eggs were identically inseminated (one drop of 1:25:7.5 c.c.) in (*a*) normal sea-water, (*b*) in sea-water to which one part of copper chloride in 500,000 parts of sea-water had been added, (*c*) in egg-water of 1,600 agglutinating units to which also one part of copper chloride in 500,000 parts of egg-water had been added. In (*a*) 100 per cent. of the eggs segmented, in (*b*) none segmented, in (*c*) 68.5 per cent. segmented. The egg-water was thus shown to protect against the copper chloride.

More elaborate experiments were then set up, one of which is given in Table IV. Here it is shown that egg-water alone

TABLE IV.

(EXP. 23.2.) IDENTICAL INSEMINATIONS IN THE FLUIDS (*a*) TO (*k*). (One drop of 1 : 25 : 7.5 c.c.)

	Per Cent. of Eggs Segmented.
(*a*) 1/500,000 Copper egg-water of 400 agglutinating units	50%
(*b*) 1/500,000 Copper egg-water of 200 agglutinating units	50%
(*c*) 1/500,000 Copper egg-water of 80 agglutinating units	50%
(*d*) 1/500,000 Copper egg-water of 40 agglutinating units	15%
(*e*) 1/500,000 Copper egg-water of 20 agglutinating units	10%
(*f*) 1/500,000 Copper egg-water of 10 agglutinating units	<0.1%
(*g*) 1/500,000 Copper egg-water of 7 agglutinating units	<0.1%
(*h*) 1/500,000 Copper egg-water of 5 agglutinating units	0
(*i*) Sea-water alone. Control 1	80%
(*j*) 1/500,000 Copper sea-water. Control 2	0
(*k*) Egg-water alone (400 units). Control 3	50%

reduces the percentage of cleavage from 80 per cent. (i, control 1) to 50 per cent. (k, control 3); that 1/500,000 copper chloride in sea-water completely inhibits fertilization (j, control 2), and that egg-water protects completely against this concentration of copper chloride down to 80 units (a, b, and c), and that its protective action at lesser concentrations falls off to zero (d, e, f, g, h).

7. *Effects of H and OH Ions on Copper Inhibition.*

Two series of experiments (Nos. 24 and 25) were run to see if increase in H or OH ions protected against the inhibitory action of copper chloride. As might have been expected, increase in acidity of the sea-water had no favorable effect. On the alkaline side some protective action is found between about 1/2,500 N NaOH and 1/666 N NaOH by volume. In the best case about 33 per cent. of the eggs segmented when 1/666 N NaOH was added to 1/500,000 copper chloride sea-water. The membranes were, however, very narrow, and the cells tended to separate in cleavage owing to this fact. The appearance of the eggs was similar to eggs segmenting in a copper solution too weak to produce complete inhibition. The effect was no doubt due to precipitation of some of the $CuCl_2$ as $Cu(OH)_2$, but the dissociation of the copper salts was still sufficient to inhibit very greatly.

8. *The Protective Action of Gum Arabic and Gelatin Against Copper.*

Egg-water stands by no means alone in protecting against the inhibiting action of copper on fertilization. The same result may be obtained with either gum arabic or gelatin, and presumably with other colloids and proteins.

The protective action of gum arabic begins at 0.2 per cent. and becomes complete at 0.8 per cent. (see Table V.). As the gum arabic has no deleterious action of its own it furnishes complete protection at the proper concentration, as the table shows.

TABLE V.

(EXP. 32.) PROTECTIVE ACTION OF GUM ARABIC. Identical inseminations in the fluids (*a*) to (*e*) and their controls. (One drop of 1 : 25 : 7.5 c.c.)

One Part of $CuCl_2$ to 500,000 Parts of	Per Cent. of Eggs Segmented.	Same Solutions Without $CuCl_2$ (Controls).	Per Cent. Segmented.
(*a*) 0.2% gum arabic in s.w.....	10%	(*a*) 0.2% gum arabic in s.w.....	100%
(*b*) 0.4% gum arabic in s.w.....	55%	(*b*) 0.4% gum arabic in s.w.....	100%
(*c*) 0.8% gum arabic in s.w.. ..	100%	(*c*) 0.8% gum arabic in s.w.....	100%
(*d*) 1.6% gum arabic in s.w.....	100%	(*d*) 1.6% gum arabic in s.w.....	100%
(*e*) Sea-water (copper control)..	0	Sea-water only.....	100%

The protective action of gelatin begins at 0.008 per cent. and becomes complete at 0.064 per cent. (see Table VI.).

TABLE VI.

PROTECTIVE ACTION OF GELATIN (EXP. 34). Identical inseminations in the fluids (*a*) to (*j*) and their controls. (One drop of 1 : 25 : 7.5 c.c.)

One Part of $CuCl_2$ to 500,000 Parts of	Per Cent. Segmented.	Same Solutions Without Copper (Control).	Per Cent. Segmented.
(*a*) Sea-water (control)	0	(*a*) Sea-water (control)	100%
(*b*) 0.0001% gelatin in s.w.	0	(*b*) 0.0001% gelatin in s.w.	90%
(*c*) 0.001% gelatin in s.w.	0	(*c*) 0.001% gelatin in s.w.	90%
(*d*) 0.002% gelatin in s.w.	0	(*d*) 0.002% gelatin in s.w.	100%
(*e*) 0.004% gelatin in s.w.	<1%	(*e*) 0.004% gelatin in s.w.	100%
(*f*) 0.008% gelatin in s.w.	20%	(*f*) 0.008% gelatin in s.w.	100%
(*g*) 0.016% gelatin in s.w.	50%	(*g*) 0.016% gelatin in s.w.	100%
(*h*) 0.032% gelatin in s.w.	90%+	(*h*) 0.032% gelatin in s.w.	100%
(*i*) 0.064% gelatin in s.w.	100%	(*i*) 0.064% gelatin in s.w.	100%
(*j*) 0.1% gelatin in s.w.	100%	(*j*) 0.1% gelatin in s.w.	100%

Thus both gum arabic and gelatin protect against the inhibiting effect of copper. Presumably any non-injurious substance that would form a non-dissociable compound with copper, thus removing it from the sphere of action of fertilization, would similarly protect. The question then arises whether the protective action of egg-water is a special instance of the general colloid (gum arabic) effect, or of the protein (gelatin) effect. I think we may say definitely that the action of egg-water is not a general protein effect, for even the strongest egg-water does not give any certain protein reaction. It is moreover practically certain that diluted egg-water which protects does not have a colloid content equivalent to the protective minimum of the gum arabic solution. There is something in the egg-water that does not give protein reaction, but with probably an equal or greater avidity for copper. There is also something in the cortex of the egg itself with a similar affinity for copper, viz.: the activable substance. It is reasonable to suppose that these two things are identical.

It is an interesting fact that egg-water also possesses a deviating effect on the inhibiting action of species-blood in fertilization which is not a general colloid effect nor yet a general protein effect (Lillie, 1914). As egg-water protects against two such different forms of inhibitor, it is reasonable to suppose it is able to do so by possessing the same substance as that on which the

inhibitors work in the egg. No other hypothesis possesses the simplicity of this one, which means that the activating substance of the egg (fertilizin) is present in the egg-water.

9. *The Effect of Copper on Activation by Butyric Acid.*

If copper chloride inhibits fertilization by combining with the fertilizin of the egg it should also inhibit activation of the egg by parthenogenetic agents as, *e.g.*, butyric acid. This consequence of the theory was found to be true. If unfertilized eggs of *Arbacia* are placed in 50 c.c. sea-water + 2 c.c. *N*/10 butyric acid, and transfers are made to 1/500,000 copper sea-water and to sea-water for control, after 30 seconds and 45 seconds, it is found that in the sea-water good to fair membranes form on practically all of the eggs in about two minutes; but in the 1/500,000 copper sea-water no membranes form and the eggs appear entirely unchanged for at least twenty minutes. Then a gradual cytolysis begins to set in, entirely similar to the cytolysis that occurs in copper sea-water without previous exposure to butyric acid after two to three hours. The butyric acid has hastened the appearance of the copper cytolysis; but the copper has entirely inhibited the typical membrane-forming reaction.

Copper present with the butyric acid does not, however, inhibit the membrane formation after transfer to sea-water. In this reciprocal experiment one part of copper chloride is added to 500,000 parts of the butyric acid solution, and the eggs receive the normal exposure. When transferred to sea-water, membranes form just as though the copper had not been present. Thus, whatever the pre-activation effect of the butyric acid may be, copper does not inhibit it, but operates only to prevent the activation of the fertilizin.

Similarly in fertilization the preliminary effect of the spermatozoön in preparing the fertilizin for discharge ("latent period"; see p. 141) is not presumed to be affected by the copper but only the actual discharge (*i.e.*, activation) of the fertilizin.

10. *A Comparison of the Effects of Mercuric Chloride.*

It will be desirable to compare the effects of the salts of other heavy metals on fertilization, and some experiments have been begun along this line. However, in this paper, mercury alone will be considered.

The effect of mercuric chloride ($HgCl_2$) on fertilization is pronounced, but it is very different from that of copper chloride: the initial stages of fertilization are relatively little affected, and the susceptibility increases as fertilization progresses; fertilized eggs show the effects much more rapidly than unfertilized. Mercury also suppresses the movements of the spermatozoa at great dilution, and offers in this way another contrast to copper.

Table VII. records an experiment in which eggs were inseminated in various dilutions of $HgCl_2$ in sea-water, between one part in 1,250,000 and one part in 15,625. Most of the eggs form fertilization membranes in the four lowest concentrations. But the eggs do not segment, except at the lowest concentration tested, and then only 20 per cent. irregularly. The fertilized eggs cytolyze more rapidly than the unfertilized.

TABLE VII

EXPERIMENT 31A. EGGS INSEMINATED IN SERIES OF $HgCl_2$ SOLUTIONS IN SEA-WATER. (One drop of 1 : 25 : 7.5 c.c.)

$HgCl_2$ in Sea-water.	Membranes.	Cleavage.	Cytolysis Begins.	Action on Sperm.
1. 1/1,250,000 . . .	100% good	20% irreg.		Faint movement only in 3 min.
2. 1/625,000	100% good	None		Same
3. 1/312,500	90%+ good	None	68 minutes	Same
4. 1/156,250	90%+ good	None	Bad in 1 hour	Paralyzed, 3 min.
5. 1/125,000	20% good 80% none	None	Fert. eggs cytolyze in 20 minutes	Paralyzed in few seconds
6. 1/62,500	15% good 85% none	None	Same as above	
7. 1/31,250	2% 98% none	None		
8. 1/15,625	None	None		Paralyzed instantly
9. Sea-water control	100% good	100%		

It was also observed that unfertilized eggs form membranes in solution 5 (Table VII.) beginning at about five minutes. When transferred to sea-water such eggs undergo changes similar to eggs treated with butyric acid.

Mercury thus exhibits an extraordinary contrast to copper: in certain comparable concentrations it paralyzes sperm, copper does not; it produces membrane formation alone, and favors it in fertilization, copper inhibits membrane formation; it suppresses cleavage and cytolyzes fertilized eggs rapidly; copper is neutral during the same stages.

The differences in the actions of copper and mercury respectively on fertilization may be explained by differences in intensity of action on the early and the later stages of fertilization respectively. In the case of mercuric chloride I have found that the inhibiting action in fertilization (which occurs at higher concentrations than in the case of copper, as for instance in solutions 5 and 6 of Table VII.) is reversible, like copper. Specifically, eggs that remain unfertilized in these solutions may be fertilized after return to sea-water, if the exposure is not too long (about 20 minutes). Presumably, therefore, mercury acts like copper in the initial stages of fertilization, though a higher concentration is required; but in the later stages mercury acts deleteriously in much lower concentrations than copper; one part of $HgCl_2$ in 625,000 parts of sea-water will completely inhibit cleavage of fertilized eggs, whereas it requires about one part of $CuCl_2$ in 62,500 parts of sea-water to produce comparable effects.

The comparison may be tabulated as follows according to the stages of fertilization:

1. Agglutination of the spermatozoön to the egg.
2. Latent period.

   Copper intervenes, Hg not, or Cu > Hg.
3. Combination of sperm receptors with fertilizin.
4. Activation = combination of fertilizin with egg receptors.
5. Sterilization = "wave of negativity."
6. Spermatozoön enters the egg.
7. Membrane formation.
8. *Hg > Cu in later events.*

It is well known that the salts of heavy metals have a powerful "poisonous" effect on enzymes. McGuigan (1904) determined that for diastase the order of poisonous effects (complete inhibi-

tion) is $N/100{,}000$ silver (nitrate), $N/33{,}333$ gold (chloride), $N/30{,}000$ mercury (chloride), $N/8{,}333$ copper (chloride). v. Euler and Svanberg (1920) studied the effects of heavy metals on the inversion of cane sugar by saccharase, and determined among many other things that mercury has about one thousand times the poisonous effect of copper; the metallic ions in question enter into combination with some constituent of the enzyme solution.

The order of poisonous effects of mercury and copper on the events of fertilization following membrane formation is thus the same as that of the poisonous action of these metals on enzymes. The concentrations also are of comparable magnitude (cf. McGuigan's data above). v. Euler and Svanberg found the range for $HgCl_2$ between complete inhibition of the ferment and no effect to lie between 1 part in 116,600 and 1 part in 1,166,000 of the solution (compare Table VII. of this paper). There is thus a rather surprisingly close agreement in the effective concentrations; so that it is reasonable to conclude that the effect of mercury and copper on fertilization following membrane formation may be due to enzyme poisoning.

The inhibiting effect of mercury and copper on the initial stages of the fertilization reaction is in the inverse order, and does not, therefore, correspond so well to the enzyme analogy. The results emphasize the strong contrast between the initial and the subsequent events of the fertilization process, which is found also in the phenomena of specificity (Lillie, 1919, 1921), and in other phenomena of fertilization.

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